Point by Point Response to Editorial and Reviewer Critiques

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you. We have done our best to ensure that there are no spelling or grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Done.

3. Please define all abbreviations during the first-time use.

Done.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Done.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Done.

6. The Protocol should contain only action items that direct the reader to do something.

Done.

7. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Done.

8. Please ensure you answer the "how" question, i.e., how is the step performed?

Done.

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done.

10. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done.

11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Done.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Done.

13. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
14. Please do not abbreviate the journal titles in the reference section.

Done.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Leaders in this field are going to share with other investigators what they've discovered about FACs sorting stromal vascular cells.

Major Concerns:
none

Minor Concerns:
none

We thank the reviewer for taking the time to review our manuscript.

Reviewer #2:

The stromal-vascular fraction of white adipose tissue is highly heterogeneous. The authors here describe a method for the isolation of functionally distinct adipose progenitor subpopulations from murine intra-abdominal adipose depots, i.e. fibro-inflammatory progenitors and adipocyte precursor cells. These two cell populations can be isolated by FACS or biotinylated antibody-based immunomagnetic bead technology. The manuscript is well prepared and the protocol is described in great detail. I have no doubt that these methods will greatly help understand better the molecular mechanisms regulating adipogenesis and intra abdominal adipose tissue remodeling in health and disease. I only have minor suggestions to make:

We thank the reviewer for taking the time to carefully review the manuscript.

1) The results show examples of APC enriched genes. It would be helpful to show FIP specific/enriched genes as well

The data presented in the current paper show the expression of adipocyte-selective genes/markers within cultured subpopulations following adipogenesis. These data are meant to illustrate the difference in adipogenic capacity. We refer readers to the original research article about these cell populations for a detailed analysis of FIPs/APCs gene expression profiles. To help investigators, we have now incorporated a table in the manuscript with a list of genes enriched in FIPs and APCs along with the respective primer sequences. These genes can be used as markers of the sorted populations.

2) The authors should discuss the strengths and weaknesses of the 2 methods they describe. Would the authors recommend one or the other depending on different downstream applications? Are there big differences in terms of cost, time, quality/purity?

This is an important point. We have now revised the discussion section to better discuss this issue.

3) At what temperature are performed the centrifugation steps?

We regret the omission of this important detail. Centrifugation was performed at 4C. We have added the centrifugation temperature in the protocol.

4) The authors use 6-8 week old mice. The authors should discuss if mice of different ages can be used, or if the protocol works better in 6-8 week old mice

This is a great question. We recommend using younger mice that are about 6-8 weeks old for better yield of APCs and FIPs. We have observed that the frequency of PDGFRβ+ APCs decrease as animals get older. Isolation of APCs from older mice may yield fewer cells. We have now added this note to the protocol and thank the reviewer for pointing this out.

5) Step1.2: "combine 2-4 fat depots": Is it different depots from one mouse or the same depot from several mice? Is there any downside to pool tissues from different mice?

We use gonadal white adipose tissue in this protocol for isolation of FIPs and APCs. We isolate two depots from one mouse (left and right) and combine up to four depots from two mice in one tube of 10ml digestion buffer. There is no downside of pooling tissue as long as the mice are of similar age and same sex. We do not recommend combining more than 4 depots in 10ml digestion buffer to avoid cell saturation/clogging of filters. However, several mice and multiple tubes of digestion buffer may be used and cells may be combined at the end of the isolation for further assays. We have now added this note to the protocol.

6) Are these methods specific to mice? The authors should discuss which species the techniques can be used with

This is a good question. At present, we can only conclude that these isolation methods and in vitro culture conditions discussed here can be used for murine intraabdominal adipose depots only. We have not tested this protocol with rat adipose tissue. Importantly, humans do not have an apparent LY6C ortholog; therefore, this particular separation strategy cannot be used with human WAT. We have now added this point to the discussion section of the manuscript and thank the reviewer for raising this issue.

Reviewer #3:

GENERAL COMMENTS

The article by Peics and collaborators describes two methods, one using fluorescence-activated cell sorting and the other using immunomagnetic beads, allowing isolation of two distinct cell populations from the stromal-vascular cell fraction of adipose tissue. Taking advantage of the specific cell-surface antigen signature, the authors were able to isolate fibro-inflammatory precursors (LY6C+, PDGFRb+, CD45-, CD31-) and adipocyte precursors (CD45-, CD31- , LY6C-, PDGFRb+, CD9-). The methodology used for this protocol description is not novel. What is original is its application to the separation of cell fractions from adipose tissue. However, more proof would be required to ascertain that the phenotype of the cells isolated is consistent with the label claimed by the Authors. Specific comments are provided below.

MAJOR COMMENTS

1- As mentioned above, more phenotypic characterization would greatly reinforce the relevance of the cell type separation proposed here. Additional experiments would be required on APC cells.

2- Bright-field microscope pictures suggest that APC accumulate intracellular lipids. However, cells are not stained and no quantification is provided. We suggest Oil Red O staining in both APC and FIP with red-pixel quantification.

We thank the reviewer for taking the time to review the manuscript and to offer constructive feedback. The focus of this manuscript is to provide a detailed method for separation of two stromal subpopulations that have been described in our prior publication. A detailed characterization for the functional properties of these cells has already been performed, peer-reviewed, and published in Hepler et al. *Elife* 2018. Given the scope of a methods paper for JoVE, we refer to the published literature regarding the characterization of these cell populations. Of note, our prior publication includes several functional assays of adipogenesis [in vitro (with ORO staining) and in vivo] and pro-inflammatory responses. In the revised manuscript, we direct readers to this prior publication for further insight into the functional properties of these cells.

3- APC seem to differentiate spontaneously. However, this might be due to the presence of insulin in the medium (ITS premix). Is the same process present when ITS is not added? What kind of medium was used on FIP in Figure 3B and 3D? To allow comparison, both cells should have received APC medium.

This is a great point. There is indeed a significant amount of insulin in the ITS premix. We have amended the discussion to clarify this point. Importantly, both APCs and FIPs did receive the same culture medium. We have amended the manuscript (figure legend and discussion section) to make this clearer.

4- How long were the cells cultivated for when PCR analyses were performed?

Cells were harvested for qPCR analysis after APCs were fully differentiated. APCs begin to undergo spontaneous differentiation as they approach confluence and take about 7-10 days to fully differentiate. FIPs were harvested at the same time as APCs. We have amended the protocol to make this clearer.

5- Similar to APC, gene expression of FIP could be investigated. Genes that could be of interest are: TNF-alpha, TGF-beta, collagen and/or metalloproteases, among others.

The data presented in the current paper show the expression of adipocyte-selective genes/markers within cultured subpopulations following adipogenesis. These data are meant to illustrate the difference in adipogenic capacity. We refer readers to the original research article about these cell populations for a detailed analysis of FIPs/APCs gene expression profiles. To help investigators, we have now incorporated a table in the manuscript with a list of genes enriched in FIPs and APCs along with the respective primer sequences. These genes can be used as markers of the sorted populations.

6- Additional experiments could include localization of FIP and APC in whole adipose tissue by immuofluorescence staining.

This is an important question; however, it is outside of the scope of this methods paper which is solely dedicated to presenting strategies to isolate the cells. We are working on developing strategies to localize the cells using commercially available antibodies. This will be included in a future publication.

7- Line 67: Excess caloric intake is not the sole factor inducing pathological remodeling of adipose tissue. Indeed, many studies show that the metabolic and functional characteristics of what is defined as 'metabolically healthy obesity' are likely to deteriorate with time.

Good point. We have amended this sentence to read, “Moreover, insulin resistance in obesity is associated with pathologic remodeling of WAT.”

8- Overall, the paper lacks many references to support some statements. For example, line 78 when describing a microenvironment that influences AT expansion; line 95 when describing FIP; line 100 when stating that APC readily differentiate into mature adipocytes.

We thank the reviewer for pointing out these omissions. We have added the appropriate references where indicated.

MINOR COMMENTS

1- Among papers that identify various cell populations in the stromal vascular fraction by single cell sequencing, it may of interest to cite the recently published paper by Vijay and collaborators (PMID: 32066997). These authors characterized and compared the stroma-vascular fraction of obese patients with type 2 diabetes vs. those without type 2 diabetes. That study could add relevance of the method proposed here.

Great point. We now make reference to this paper in the revised Discussion as we refer to the point below. We thank the reviewer for this suggestion.

2- Do the two cell populations investigated here (FIP vs. APC) exist also in human stromal-vascular fraction? In what measure are they different or similar to the ones found in humans?

This is a great question. Based on our unpublished work and our cursory analysis of published datasets, we think that cells reminiscent of the FIPs and APCs we describe are indeed present in human tissue. However, we are not yet able to draw any conclusions until we isolate the putative populations and test them functionally. This is part of an on-going effort in the lab. Importantly, humans do not have a LY6C ortholog; therefore, our

sorting strategy cannot be used to isolate APCs from human WAT. We now make this latter point clear in the manuscript.